The Positions of Normal Leukocytes on the Scattergram of the Newly Developed Abnormal Cell-detection Channel of the XN-Series Multi-parameter Automated Hematology Analyzers

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The newly launched XN-Series (Sysmex Corporation, Kobe, Japan) multi-parameter automated hematology analyzers contain a new channel — the WPC channel, which detects blasts and abnormal cells of the lymphocyte class. In this channel, dedicated WPC reagents penetrate the cell membranes of leukocytes and stain the intranuclear DNA. The forward scattered light (FSC), side scattered light (SSC), and side fluorescent light (SFL) of the cells are then measured and expressed as two 2D scattergrams, the WPC and the WPC (SSC-FSC) scattergram.

Here, we examined the WPC scattergram distribution of the 3 major subtypes of leukocytes (T-lymphocytes, monocytes, and neutrophils) isolated from healthy volunteers. We also performed some molecular analyses to elucidate the underlying reasons for lymphocyte distribution. We confirmed T-lymphocytes, monocytes, or neutrophils from healthy volunteers were located in the left, middle, or right cluster of the WPC scattergram. Moreover, T-lymphocytes were separated in 2 clusters in the WPC (SSC-FSC) scattergram, and T-lymphocytes had the lowest FSC intensity, followed by monocytes and neutrophils. Confocal laser scanning fluorescence microscopy showed that all 3 leukocyte subtypes had the same stain intensity level following incubation with WPC reagents. Transmission electron microscopy after treatment with WPC reagents revealed that the intracellular complexity was the lowest in lymphocytes, followed by monocytes, and it was the highest in neutrophils; this order was consistent with the order of SSC intensities. Additionally, the intracellular structure of T-lymphocytes showed 2 types: some cells had bulging nuclei and little cytoplasm, whereas others retained most of their cytoplasm with organelles and had dense nuclei. Scanning electron microscopy after treatment with the reagents showed that T-lymphocytes were the smallest cell type, followed by monocytes and neutrophils. Surface morphology of T-lymphocytes also showed 2 types: some cells were much smaller than control and had abnormally smooth surfaces, and others had the size and surface-structures equivalent to control cells. Finally, by using a general-purpose flow cytometer, we investigated why T-lymphocytes exhibited 2 kinds of morphology, and found that most small lymphocytes lost their surface antigen. This result suggests that T-lymphocytes with highly damaged cell membranes appear to be smaller post treatment.

In summary, the 3 major leukocyte subtypes form 3 major clusters in the WPC scattergram. From the left of the scattergram, the appearance was in the order of T-lymphocytes, monocytes, and then neutrophils. The results also show that T-lymphocytes appear in 2 clusters in the WPC (SSC-FSC) scattergram according to the level of cell membrane damage.

Key Words Multi-parameter Automated Hematology Analyzer, XN Series, WPC channel, Scattergram

INTRODUCTION

The XN-Series is a series of multi-parameter automated hematology analyzers that were developed to provide utility, operational and clinical value.The WPC channel, which is the one of the newly equipped functions, mainly detects and flags blasts and abnormal cells of the lymphocyte class by exploiting the principle of flow cytometry.

In the WPC channel, surfactant in Lysercell WPC cause hemolysis and dissolution of red blood cells and platelets, and differentially disrupts the cell membrane of white blood cells. Then, the fluorescent dye in Fluorocell WPC enters the cells and stains the nucleic acid. The forward-scattered-light (FSC), side-scattered-light (SSC), and side fluorescent light (SFL) intensities of these stained leukocytes can be measured using the principle of flow cytometry through excitation by a 633-nm laser beam. This information is subsequently expressed as two 2D scattergrams: the WPC scattergram and WPC (SSC-FSC)

scattergram (***Fig. 1***). Detection of abnormal leukocytes in the WPC channel has been confirmed using clinical samples. Abnormal leukocytes appear in clusters that are distinct from the clusters of normal leukocytes in both the WPC and WPC (SSC-FSC) scattergrams [1,4].

The overall position of leukocytes from healthy volunteers has been confirmed in the WPC and WPC (SSC-FSC) scattergrams; however, which cluster represented a lymphocyte subtype was not precisely known.

In this study, we examined and defined the position of the 3 major subtypes of leukocytes in the WPC scattergram. Seen from the left, 3 clusters of the WPC scattergram were in the order of T-lymphocytes, monocytes and neutrophils. The results also showed that T-lymphocytes appeared in 2 clusters in the WPC (SSC-FSC) scattergram according to the level of cell membrane damage.

MATERIAL AND METHODS

1. Samples

Peripheral blood from 16 healthy human subjects was collected in tubes containing EDTA-2K by venipuncture (Terumo Corporation, Tokyo, Japan). Subjects gave their informed written consent before participation.

2. Cell separation

1) Density-gradient centrifugation

Peripheral blood from healthy volunteers was diluted in the same volume of PBS. According to the manufacturer's instructions, blood was overlaid on 2 types of Lymphocyte Separation Solution, with d = 1.077 and d = 1.119 (Nacalai Tesque, Inc., Kyoto, Japan), and the mononuclear cell-rich and granulocyte-rich fractions were prepared by density-gradient centrifugation and washed with PBS.

2) Magnetic cell sorting

T lymphocytes, monocytes, and neutrophils were isolated from the mononuclear cell-rich fraction by negative selection with the RoboSep system (STEMCELL Technologies Inc., British Columbia, Canada) according to the manufacturer's instructions. Then, in order to recover the morphology observed in the peripheral blood, isolated cells were cultured in 1% BSA/PBS for 1 h at 37°C.

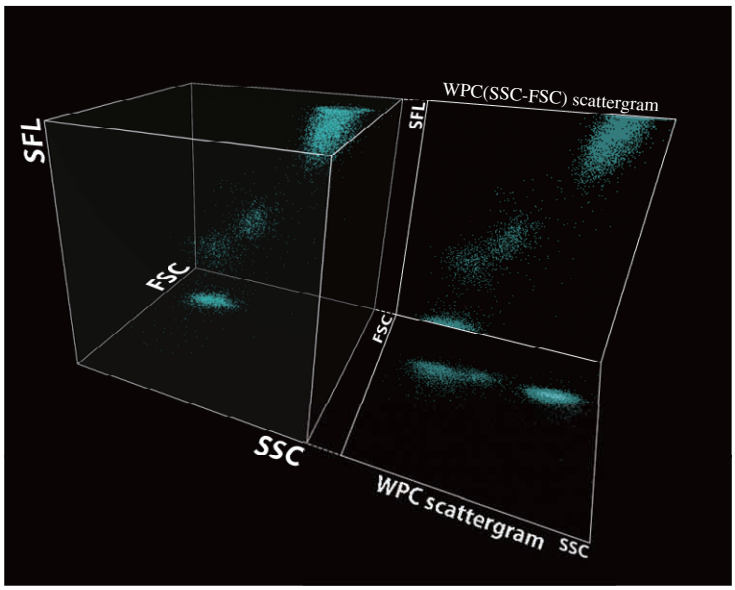

Fig. 1 *WPC scattergram and WPC (SSC-FSC) scattergram*

In the WPC channel, the strength of forward scattered light (FSC), side scattered light (SSC), and side fluorescent light (SFL) of the cells was measured and expressed as two 2D scattergrams: the WPC scattergram and the WPC (SSC-FSC) scattergram. Left expressed as a 3D scattergram with 3 axes representing SSC, FSC, and SFL. Data was obtained from normal leukocytes isolated from healthy volunteers.

3. Confirmation of the purification of isolated cells

Isolated T lymphocytes, monocytes, and neutrophils were incubated with FITC-conjugated monoclonal antibodies to CD3 (T lymphocyte marker), CD14 (monocyte marker), and CD16b (neutrophil marker); all antibodies were from Beckman Coulter, Inc., (Boulevard, USA) and used at a concentration of 20 mg/L in PBS. The cells were incubated for 20 min at 4°C in the dark. FITC-conjugated mouse IgG_1 antibody (DAKO) was used as a negative control. Stained cells were washed with PBS and analyzed using a FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ, USA), and the purity of isolated cells was then verified.

4. Measurement by XN-Series

Peripheral blood and isolated cells were measured using the XN-2000 multi-parameter automated hematology analyzers, and their position on the WPC scattergram and WPC (SSC-FSC) scattergrams was then confirmed.

5. Treatment with WPC-specific reagents

Isolated cells were treated with WPC-specific reagents (Lysercell WPC, Fluorocell WPC, Sysmex Corporation, Kobe, Japan), with the dilution rate and reaction time similar to those used with the XN-2000 analyzer.

6. Confocal laser scanning microscopy (CLSM) analysis

Stained cells were immediately attached to poly-L-lysine (SIGMA Corporation, Missouri, USA)-coated coverslips and observed using a confocal laser scanning system (IX81; Olympus Corporation, Tokyo, Japan; CSU-X1, Yokogawa Electric Corporation, Tokyo, Japan; ImagEM, Hamamatsu Photonics K.K, Hamamatsu, Japan).

7. Electron microscopy analysis

1) Cell fixation

Each cell preparation was fixed in a 1.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) solution in PBS for at least 16 h at 4°C.

2) Examination by transmission electron microscopy (TEM)

Fixed cells were attached to MAS-coated glass slides using Cytospin (Thermo Fisher Scientific, MA, USA) and post-fixed in 1% osmium tetroxide for 45 min at room temperature (RT). Following osmium fixation, the samples were dehydrated in a graded series of ethanol and inversion-embedded in Quetol 812 (Nisshin EM Corporation, Tokyo, Japan). The samples were cut into 60-80 nm sections with an Ultracut UCT ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Fixed cells were observed with the H-7500 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

3) Examination by scanning electron microscopy (SEM)

Fixed cells were attached to poly-L-lysine-coated glass slides. After fixation and dehydration using the same method as described above for TEM, ethanol was replaced with *t*-butyl alcohol (Wako Pure Chemicals, Osaka, Japan). The samples were then dried using a freeze-dryer (Hitachi High-Technologies) and osmium-coated using Neoc (MEIWAFOSIS, Tokyo, Japan). The samples were then observed using a JSM-7500F system (Nippon-Denshi, Osaka, Japan).

8. Analysis of T lymphocytes

1) Representation of WPC scattergram using general-purpose flow cytometer.

Peripheral blood samples were stained with WPC-specific reagents according to Procedure 5, described above. Using a general-purpose flow cytometer, stained samples were represented on the WPC scattergram, considering SSC as the X-axis and SFL as the Y-axis. On the WPC (SSC-FSC) scattergram, SSC was plotted on the X-axis and FSC, on the Y-axis (***Fig. 5***).

2) Analysis of T lymphocytes

Leukocytes isolated according to Procedure 2 described above were stained with specific antibodies and negative controls according to Procedure 3. Double staining was performed according to Procedure 5, and cells were analyzed by general-purpose flow cytometry.

RESULTS

1. Position of the isolated leukocyte subtypes

Isolated cells derived from peripheral blood from healthy volunteers were measured using an XN-2000 analyzer, and their position on the WPC scattergram was examined. This involved isolation of the 3 major subtypes of leukocytes (T-lymphocytes, monocytes, and neutrophils) from peripheral blood by using density-gradient centrifugation and magnetic cell sorting [7,8].
General-purpose flow cytometry revealed that the purity of the isolated T-lymphocytes, monocytes and neutrophils were in the order of 95.6%, 57.6% and 97.8%, respectively (***Fig. 2A***). Subsequently, cells were measured using the XN-2000 device, and their position on the WPC scattergram was compared with that of leukocytes from whole peripheral blood. Leukocytes of whole blood were detected on the WPC scattergram as 3 clusters, termed in this study as left, middle, and right (***Fig. 2B***, whole blood). The position of the isolated T-lymphocytes, monocytes, and neutrophils on the WPC scattergram corresponded to the clusters on the left (K), middle (M) and right (N), respectively. On the other hand, in the WPC (SSC-FSC) scattergram of whole blood, normal leukocytes were detected as 4 clusters. Isolated T-lymphocytes corresponded to the position of the upper (L1) and lower (L2) left cluster, and monocytes and neutrophils were located in the middle (M) and right cluster (N) of whole blood, respectively (***Fig. 2C***).
In conventional flow cytometry, FSC reflects the information of the particle size, whereas SSC represents information on the cell morphology and inner particles (e.g., nucleus and cellular granules). Additionally, in the case of normal leukocytes from healthy volunteers, WPC-specific reagents are known to stain nuclear DNA, so that SFL reflects the DNA content of the cell. Damage to the cell membrane is of the same level for all leukocytes from healthy volunteers that were treated with WPC-specific reagents.
These data allow us to make the following conclusions regarding leukocytes treated with WPC-specific reagents.

1) There is little difference in fluorescence intensity following staining with WPC-specific reagents
2) The intracellular structure complexity as determined by staining with reagents is in the following order:
   T-lymphocytes < monocytes < neutrophils
   (T-lymphocytes having the simplest structure).
3) The size of cells determined by staining with reagents is in the following order:
   T-lymphocytes < monocytes < neutrophils
   (with T-lymphocytes being the smallest).
4) T-lymphocytes are segregated into 2 clusters of different sizes.

We then validated these 4 findings using CLSM, TEM, SEM, and general-purpose flow cytometry.

2. The fluorescence intensity of each subtype of leukocytes after treatment with WPC-specific reagents

Each subtype of leukocytes isolated from the peripheral blood of healthy volunteers was treated with WPC-specific reagents. Using CLSM, we then confirmed that mainly the nuclei of these cells were stained (***Fig. 3A-C***). From the microscopic images, we analyzed the intensity of fluorescent staining on a per-cell basis. When the fluorescence intensity of T-lymphocyte was set at 1.00, the average fluorescence intensity value and standard deviation were 1.07 ± 0.14 for neutrophils (***Fig. 3 A-C***). These results corresponded with those of monocytes (1.02 ± 0.19), with little difference observed in the fluorescence staining intensity of cells treated with the WPC-specific reagents (***Fig. 2, Fig. 3D***). Together with the fact that there is little difference in the DNA content between different subtypes of leukocytes, this finding led us to conclude that WPC-specific reagents mainly stain DNA.

3. The morphology of each leukocyte subtype following treatment with WPC-specific reagents

Each subtype of leukocytes isolated from the peripheral blood of healthy volunteers was treated with WPC-specific reagents under the same conditions in the XN analyzers. Samples were then fixed, cut, electron-stained, and observed by TEM (***Fig. 4A-C***). As a result, T-lymphocytes could be classified into 2 types; one which had lost most of its cytoplasm and shrank overall in size (***Fig. 4A***, △), and another type, which had comparatively retained most of its cytoplasm and organelles and had well-packed nuclei (***Fig. 4A***, ▲). Monocytes retained their cytoplasm and intracellular granules, as well as retained a more complex intracellular structure than T-lymphocytes as a whole (***Fig. 4B***). Neutrophils retained the most complex intracellular structure among the 3 subtypes, as they contain bilobular nuclei and many characteristic granules (***Fig. 4C***). The results of the TEM observation corresponded with the data presented in ***Fig. 2***, in that intracellular structure was least complex in T-lymphocytes, followed by monocytes and neutrophils. Moreover, T-lymphocytes could be segregated into 2 types according to their size. Fixed samples were coated with osmium and observed by SEM for their surface structure (***Fig. 4 D-F***). This revealed that cell size increased from T-lymphocytes (smallest) to monocytes and neutrophils (largest). SEM analysis also confirmed that T-lymphocytes were of 2 types. One was a small cell type that had lost its original surface structure, and the other was the large cell type, which maintained their original surface structure. These SEM observation results corresponded with the suggestion from the result of ***Fig. 2***.
These electron microscopic observations suggest that monocytes and neutrophils appear to retain their original intracellular structure upon staining. However, T-lymphocytes behave differently: one subset loses most of its cytoplasm because of cell membrane loss and bulging nuclei, whereas another subset retains original intracellular structure.

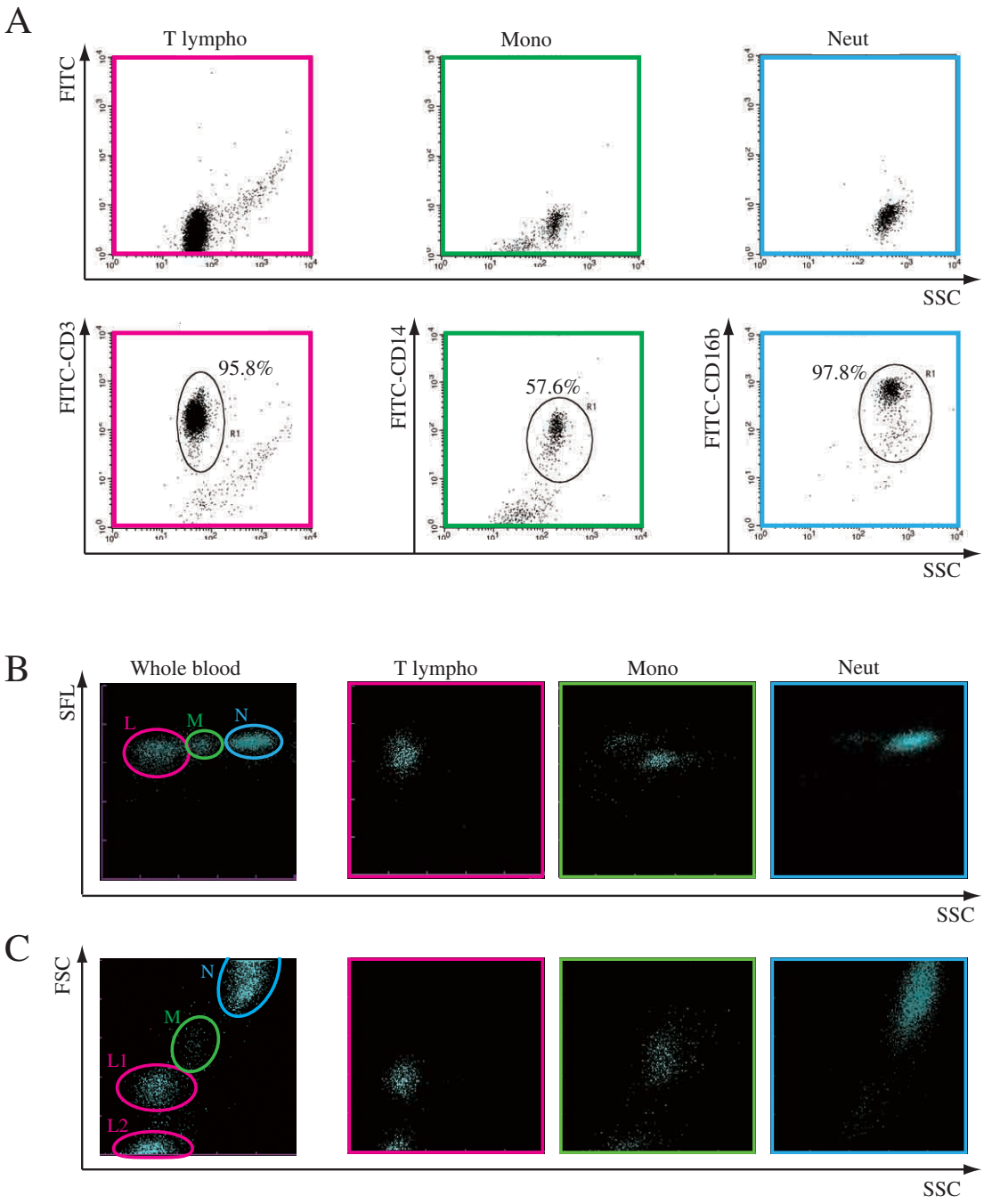

Fig. 2 *The positions of 3 main subtypes of leukocytes isolated from the peripheral blood of healthy volunteers*

(A) Purity check of isolated leukocytes by FACSCalibur
Upper: each subtype was stained and compared to the FITC-conjugated negative control via analysis with FACSCalibur
lower: each subtype of leukocyte was stained with a specific monoclonal antibody (T-lymphocytes, anti-CD3 antibody; monocytes, anti-CD14 antibody; neutrophils, anti-CD16 antibody).
(B) WPC scattergram of whole blood and subtype fraction of each leukocyte
WPC scattergram of whole blood isolated from healthy volunteers and subtype fraction of each leukocyte isolated from whole blood
Left, whole blood; left-middle, T-lymphocytes; middle, monocytes; right, neutrophils
(C) WPC (SSC-FSC) scattergram of whole blood and subtype fraction of each leukocyte
WPC (SSC-FSC) scattergram of whole blood isolated from healthy volunteers and 3 main subtype fraction of each leukocyte isolated from whole blood
Left, whole blood; left-middle, T-lymphocytes; middle, monocytes; right, neutrophils

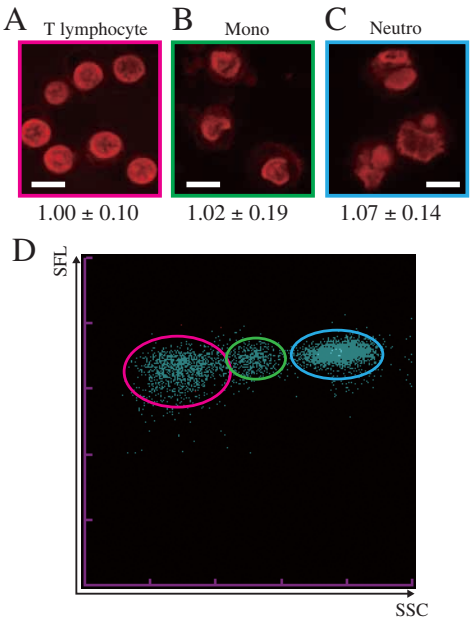

Fig. 3 *Fluorescence intensities and images of each subtype of leukocytes stained with WPC-specific reagents*

(A-C) Confocal laser scanning microscopy images of each isolated subtype of leukocytes treated with WPC-specific reagents
A) T-lymphocytes, B) monocytes, C) neutrophils
The numbers under the panel are the average intensities and standard deviation of fluorescence, where the fluorescence intensity of T-lymphocytes is set at 1.0 Bar= 5μm
D) WPC scattergram of whole blood isolated from healthy volunteers
T-lymphocytes, monocytes, and neutrophils are indicated by magenta, green, and blue, respectively.

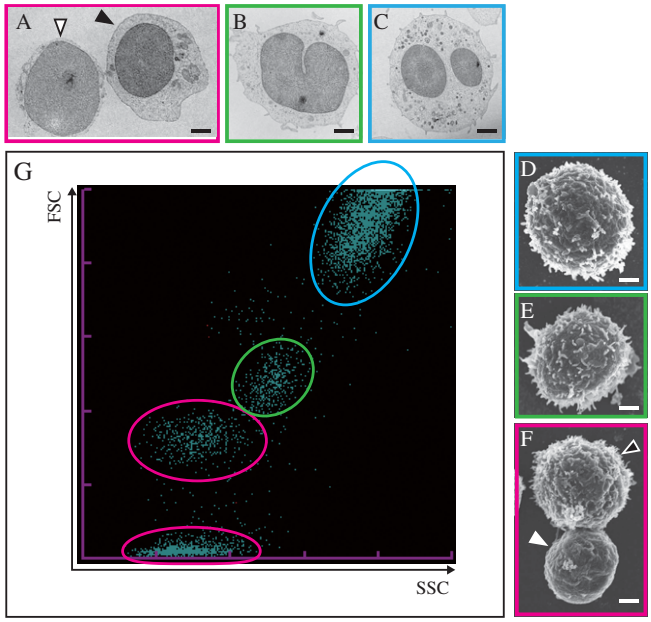

Fig. 4 *Electron microscopy images of each subtype of leukocytes treated with WPC-specific reagents*

A-C) Transmission electron microscopy images of isolated subtypes of leukocytes treated with WPC-specific reagents
A) T-lymphocytes, B) monocytes, C) neutrophils
D-F) Scanning electron microscopy images of isolated subtypes of leukocytes treated with WPC-specific reagents
D) neutrophils, E) monocytes, F) T-lymphocytes
▲ , △ of T-lymphocytes indicates whether the degree of damage to the cells was strong or weak, respectively; Bar=1μm
G) WPC (SSC-FSC) scattergram of whole blood obtained from healthy volunteers. T-lymphocytes, monocytes, and neutrophils are indicated by magenta, green, and blue, respectively.

4. Mechanisms underlying the emergence of 2 T-lymphocytes subsets

We next explored potential reasons that might explain the morphology of the T-lymphocytes treated with WPC-specific reagents, as discussed above.

The CD3 antigen, which is specifically expressed on T-lymphocytes, is a part of a large complex, including the receptor of T-cell antigen [9]. It is a single-pass protein that does not strongly bind to intracellular structures; it is therefore readily lost from the lipid bilayer membrane when it is perturbed.

T-lymphocytes isolated from healthy volunteers were stained with FITC-conjugated anti-CD3 antibody treated with WPC-specific reagents, resulting in an SSC-fluorescence intensity dependent on FITC (***Fig. 5D***), SSC-fluorescence intensity and SSC-FSC (representation of WPC (SSC-FSC) scattergram; ***Fig. 5F***) dependent on WPC reagents (representation of WPC scattergram; ***Fig. 5E***),. Cells were then examined to determine which clusters in the WPC scattergram corresponded to those that maintained the CD3 antigen.

As a result, T-lymphocytes, which lost most of their CD3 (***Fig. 5D, R1***), were strongly stained with WPC-specific reagents (***Fig. 5E, Red***) constituted a lower cluster of FSC (***Fig. 5F, Red***) and a somewhat lower cluster of SSC (***Fig. 5E,F***) compared to the T-lymphocytes that maintained CD3 (***Fig. 5D, R2***). As mentioned previously, the presence or absence of CD3 reflects the degree of damage to the cell membrane. It was suggested that the high FSC position was the cluster of cells that maintained their original morphology, and the low FSC position was the cluster that lost most of their cell membrane and underwent shrinkage.

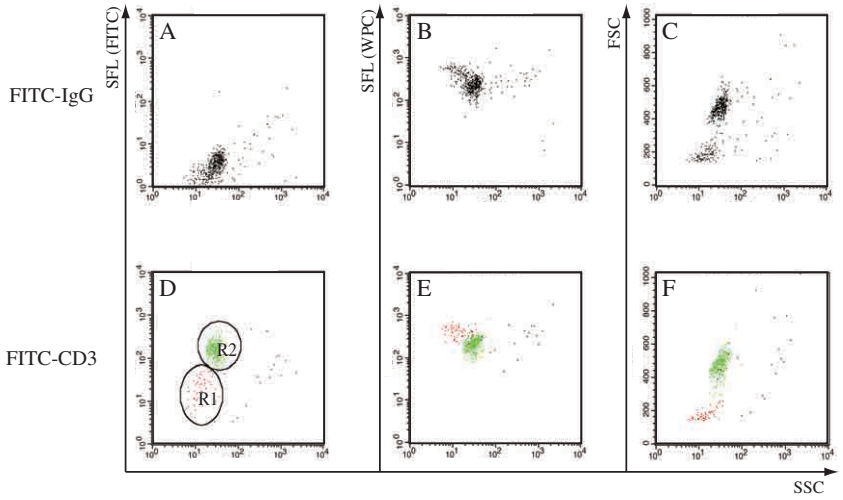

Fig. 5 *Analysis of T-lymphocytes isolated from peripheral blood of healthy volunteers*

T-lymphocytes isolated from peripheral blood of healthy volunteers were stained with FITC-conjugated negative control (A-C) or anti-CD3 antibody (D-F), and double-stained with WPC-specific reagents, and then analyzed by FACSCalibur.
(A, D) Confirmation of the staining with FITC-conjugated antibody
(B, E) Representation of the WPC scattergram by FACSCalibur
(C, F) Representation of the WPC (SSC-FSC) scattergram by FACSCalibur
Fluorescence intensity of FITC in D scattergram: low(R1), red; high(R2), green; and E and F are indicated by the same color.

DISCUSSION

In this study, we examined where each of the 3 major subtypes of leukocytes from healthy volunteers were positioned in the WPC scattergram. We also studied the reasons why they were located there.

There was little difference in SFL intensity following staining with WPC-specific reagents between the 3 main subtypes of leukocytes, as the dye mainly stained their nuclei (***Fig. 3***). Our data are supported by other results that show normal peripheral blood mature leukocytes from healthy volunteers usually have the same amount of nuclear DNA [5].

We also observed that SSC intensity in the WPC scattergram was the lowest in T lymphocytes, followed by monocytes and then neutrophils. This result corresponds with the TEM observation that T cell intracellular complexity is the lowest and neutrophil complexity is the highest. It was hypothesized that the original organelle content of cells would affect their detergent tolerance [6]. This may explain why cells appear in distinct clusters in our plots because the complexity of each leukocyte subtype varies.

However, malignant abnormal lymphocytes have a high intensity of SFL in the WPC scattergram. (***Fig. 6A***) This may be because malignant abnormal lymphocytes have more apparent DNA compared with normal leukocytes, as they rapidly proliferate and many cells may appear as double bodies [7]. On the other hand, immature leukocytes have a low intensity of SFL in the WPC scattergram and a high intensity of FSC in the WPC (FSC-SSC) scattergram (***Fig. 6B***). It is reported that immature leukocytes have high detergent tolerance [8]. This previous finding suggests that the reagent cannot easily enter the cell, but instead remains in the cell membrane; thus, the cells maintain their size and original structures including organelles.

Moreover, T-lymphocytes are segregated into 2 clusters in the WPC (FSC-SSC) scattergram according to the degree of cell membrane damage. This result implies that there are 2 types of T lymphocytes: with high and low detergent tolerance. There are some reports that subtypes of lymphocytes separated from normal peripheral blood also have different detergent tolerances [9,10]. This finding implies that 2 types of T-lymphocytes can be detected according to the degree of cell membrane damage. Furthermore, Wolf et al. have reported that activated monocytes increase their reagent tolerance and, therefore, have a higher detergent tolerance than normal leukocytes[11,12]. Thus, detergent tolerance may indirectly reflect cellular conditions such as activation.

In this study, we examined where each of the subtypes of leukocytes from healthy volunteers were observed in the WPC scattergram, and what parameters affected their distribution. In future studies, we will compare the positions of abnormal and immature cells (or experimental models of these cells) in the scattergram. We suggest that the differential response to membrane detergents may be exploited in order to distinguish target cells from other cells.

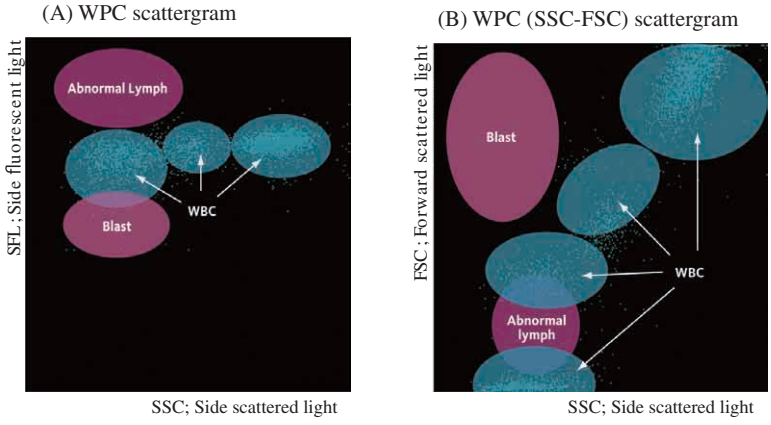

Fig. 6 *The appearance position of abnormal leukocytes in WPC channel*

(A) WPC scattergram (B) WPC (SSC-FSC) scattergram
Red cluster: blasts and abnormal lymphocytes Blue cluster: normal leukocytes
WPC channel detects the abnormal leukocytes with the proprietary algorithm
and with SSC, SFL and FSC intensity from their different reaction of WPC reagents.

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